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The Ca^{2+}-Induced Structural Changes in Troponin In-Situ and In-Vitro: A FRET Study in Permeabilized Cardiac Muscle Fibers and Reconstituted Thin Filaments

King-Lun Li[1], John R. Solaro[2], Wenji Dong[1].
[1]Washington State University, Pullman, WA, USA, [2]University of Illinois at Chicago, Chicago, IL, USA.

Ca^{2+} binding to N-domain of cardiac troponin C (N-cTnC) initiates a cascade of structural changes within the thin filament. Among these changes, Ca^{2+}-induced structural changes in troponin play a critical role in the allosteric regulation of the force-generating acto-myosin interactions. Although previous in-vitro studies have provided insight into these structural changes, how they are modulated by geometric and mechanical constraints conferred by the sarcomeric lattice structure in muscle tissue remains unknown. Recently we have developed a technique to quantitatively measure these structural changes in chemically-skinned muscle fibers using time-resolved FRET. In this study, we use this technique to monitor the Ca^{2+}-induced N-cTnC opening in skinned muscle fibers and compare the obtained results to in-vitro studies. FRET donor (AEDANS) and acceptor (DDPM) modified double-cysteine mutant cTnC(13C/51C)AEDANS-DDPM was reconstituted into skinned muscle fibers to examine the N-cTnC opening. To study the effects of crossbridge and sarcomere-length based activation on this structural change, simultaneous measurements of fluorescence intensity decays and isometric tension were performed. Our results show that at steady-state, the Ca^{2+}-induced N-cTnC opening is proportional to muscle force development, but the level of opening varies depending on the presence or absence of strong crossbridges (XB). For instance, in the presence of sodium vanadate (inhibitor of XB) no Ca^{2+}-induced force response was observed, but Ca^{2+}-induced opening of N-cTnC in the fibers was evident but with a smaller magnitude. Furthermore, when compared to in-vitro measurements, results from muscle fibers suggest that N-cTnC may adopt a more compact structure in muscle fibers due to the constraints imposed by the myofilament lattice structure. The effects of the myofilament lattice structure on structural dynamics of N-cTnC are also discussed in terms of FRET distance distributions.

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Characterization of a Cardiac Troponin C-Troponin I Chimera Protein

Sandra E. Pineda-Sanabria[1], Olivier Julien[2], Monica X. Li[1], Brian D. Sykes[1].
[1]University of Alberta, Edmonton, AB, Canada, [2]University of California, San Francisco, San Francisco, CA, USA.

Association of the regulatory N-domain of cardiac troponin C (cNTnC) with the switch region of cardiac troponin I ($cTnI_{147-163}$) is a major step in Ca^{2+} regulation of myocardial contraction by troponin as it triggers a series of downstream events that leads to contraction. Investigation of this interaction in the presence and absence of ligands, including Ca^{2+} and cardiotonic drugs, is important to understanding the regulation of cardiac contraction. This is commonly studied *in vitro* using isolated cNTnC and $cTnI_{147-163}$; however, this does not accurately reflect the *in situ* environment since cNTnC and $cTnI_{147-163}$ are spatially confined in the myofibril by being attached to their respective structural scaffold domains, the C-domain of cTnC (cCTnC) and the troponin T binding region of cTnI ($cTnI_{80-136}$). We have designed, expressed and characterized a cardiac chimera (cChimera) protein containing cNTnC linked to $cTnI_{144-173}$ through a linker containing a tobacco etch virus or thrombin protease cleavage site (similar to skeletal chimera of Tiroli *et al*, 2005). Our results show that Ca^{2+} binding to cChimera (K_D 0.15 ± 0.05 μm) is tighter than to cNTnC (K_D 2.6 ± 0.1 μm) and to that observed in skinned muscle fibers (K_D 1.2 μm). We assessed binding of the calcium sensitizer dfbp-o, and desensitizer W7, and show that cChimera is particularly useful for studying and searching interactions of cNTnC-$cTnI_{144-173}$ with potential agonists. Our cChimera also facilitates the production of the switch cTnI peptide using bacterial cultures by enzyme specific proteolysis after expression along with cNTnC. The dynamic properties of cChimera, along with Ca^{2+} and drug binding properties, make cChimera a convenient system for the investigation of regulation of troponin by small molecules that target the cTnC-cTnI interface.

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Conformation and Dynamics of the Troponin I C-Terminal Domain

Lauren Ann Metskas, Elizabeth Rhoades.
Yale University, New Haven, CT, USA.

The troponin complex acts as a molecular switch in striated muscle cells to regulate myosin attachment to and isomerization on actin filaments. Transitions between the inactive and active states of the cardiac thin filament require extensive domain movements and binding exchanges involving the C-terminal domain of cardiac troponin I (TnI-C, residues 130-210). Mutations in TnI-C are associated with hypertrophic cardiomyopathy, highlighting the importance of this domain in regulating cardiac contraction. However, the conformation and dynamics of TnI-C are poorly characterized due to order/disorder transitions between binding states, leaving mechanistic details undetermined for both wild-type and diseased complexes. TnI-C is likely disordered in solution and the unbound state [1], coupling binding and folding as it fly-casts onto its actin/tropomyosin binding site to block myosin access to actin [2].
Here we use a combination of computational and experimental techniques to probe the conformation and dynamics of TnI-C in its multiple binding states, using pairwise fluorescence measurements applicable to both the solution-state and the thin filament-docked complex. Our molecular dynamics simulations are consistent with TnI-C disorder in the unbound state; simulations beginning from a helical model unfold regardless of starting orientation or the presence of the rest of the complex, and there is no consensus structure in the disordered state over multiple 50 ns simulations of the free domain. Remarkably, the presence of the N-terminal globular head of troponin C affects TnI-C conformation *in silico*, emphasizing the importance of interdomain and intermolecular interactions in the troponin complex.

1. Julien, O., Mercier, P., Allen, C.N., Fisette, O., Ramos, C.H.I, *et al.* (2011) *Proteins* 79: 1240-1250.
2. Zhou, Z., Li, K-L, Riek, D. Ouyang, Y., Chandra, M., Dong, W.J. (2012) *J. Biol. Chem.* 287(10): 7661-7674.

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Pseudophosphorylation of Cardiac Troponin I Residues 23/24 Decreases Myofilament Ca^{2+} Sensitivity in Transgenic Mice Containing D230N Mutation in α-Tropomyosin

John J. Michael[1], Lauren Tal[2], Jil C. Tardiff[3], Murali Chandra[1].
[1]Washington State University, Pullman, WA, USA, [2]Albert Einstein College of Medicine, Bronx, NY, USA, [3]University of Arizona, Tucson, AZ, USA.

The role of sarcomeric mutations in pathological cardiac-remodeling is not well understood. Furthermore, the constant modulation of myofilament contractility by post-translational modifications (PTM) of regulatory proteins, to adapt to a frequently changing circulatory demand, adds to the complexity. Therefore, we investigated how such a PTM in cardiac troponin I (cTnI) interacted with D230N (a dilated cardiomyopathy-related α-tropomyosin mutation), to alter the contractile properties of the myofilament. We reconstituted the pseudophosphorylated form of cTnI ($cTnI_{D23/D24}$) into detergent-skinned papillary muscle fibers from non-transgenic (NTG) and transgenic D230N mice, to mimic PKA-mediated phosphorylation in response to β-adrenergic stimulation. We carried out mechanical and dynamic studies on $cTnI_{D23/D24}$-D230N and control fibers at sarcomere length 2.3 μm. The pCa-tension relationship revealed that $cTnI_{D23/D24}$ decreased the pCa_{50} ($-\log_{10}[Ca^{2+}]_{free}$ required for half maximal activation) from 5.61 ± 0.02 to 5.34 ± 0.01 in NTG fibers and from 5.49 ± 0.02 to 5.22 ± 0.01 in D230N fibers. Thus, $cTnI_{D23/D24}$ uniformly decreased pCa_{50} by 0.27 pCa units in both NTG and D230N fibers. Interestingly, D230N mutation also uniformly decreased pCa_{50} by 0.12 pCa units, regardless of the phosphorylation status of cTnI. This indicates that $cTnI_{D23/D24}$ and D230N act independently to exert an additive effect of decreasing myofilament Ca^{2+} sensitivity. Therefore, under sub-maximal concentrations of Ca^{2+}, the amount of force produced would be greatly impaired, resulting in systolic dysfunction.

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Effects of the N-Terminus of Cardiac Troponin T on Contractile Activation Depend on the Type of Myosin Heavy Chain Isoform

Ranganath Mamidi, Murali Chandra.
Washington State University, Pullman, WA, USA.

We have previously shown that two specific regions (residues 1-43 and residues 44-73) in rat cardiac troponin T (RcTnT) have divergent effects on contractile function against an α-myosin heavy chain (α-MHC) background (*Mamidi et al., Abstract: Biophys J , vol.100, Issue 3, p586a, 2010*). Because the effects of Tn isoforms are modulated by an interplay with MHC, we hypothesized that the divergent effects of residues 1-43 and residues 44-73 in cTnT would be further modulated by a shift in MHC isoform to β-MHC. This has a direct bearing on understanding the effects of alterations in cTnT and MHC isoforms not only in health but also in disease. We generated two recombinant mutants ($RcTnT_{1-43}$ and $RcTnT_{44-73}$) by deleting residues 1-43 and 44-73 in RcTnT, respectively. We studied contractile function by reconstituting $RcTnT_{1-43}$ and $RcTnT_{44-73}$ recombinant proteins into detergent-skinned papillary muscle fibers from normal rat hearts (α-MHC) and propylthiouracil-treated rat hearts (β-MHC). Our data shows that functional alterations induced by $RcTnT_{1-43}$ and $RcTnT_{44-73}$ were different in α-MHC vs. β-MHC fibers. For example, $RcTnT_{1-43}$ decreased both Ca^{2+}-activated maximal tension (by ~46%) and

myofilament Ca^{2+}-sensitivity (by 0.09 pCa units) in α-MHC fibers. However, $RcTnT_{1-43}$ modestly decreased maximal tension (by ~18%), with no effect on Ca^{2+}-sensitivity in β-MHC fibers. Thus, the desensitizing effect of $RcTnT_{1-43}$ was attenuated by β-MHC. Ca^{2+}-activated maximal tension data were supported by myofiber dynamic stiffness measurements. Another major finding is that $RcTnT_{44-73}$ abolished the sarcomere length-dependent increase in Ca^{2+}-sensitivity in β-MHC, but not in α-MHC fibers. Thus, our data demonstrates that the functional outcome of the N-terminus of cTnT is modulated by the type of MHC isoform present.

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Calponin Cross-Links Unphosphorylated Myosin to Actin and Enhances its Binding Force

Horia N. Roman[1,2], Nedjma B. Zitouni[1,2], Linda Kachmar[1,2], Gijs Ijpma[1,2], Apolinary Sobieszek[3], Anne-Marie Lauzon[1,2].
[1]McGill University, Montreal, QC, Canada, [2]Meakins-Christie Laboratories, Montreal, QC, Canada, [3]Institute for Biomedical Aging Research, Smooth Muscle Lab at the Life Science Center, Innsbruck, Austria.

Smooth muscle is unique in its capability to maintain force for long periods of time at low ATP (energy) consumption. This property, called the latch-state, is hypothesized to occur due to the dephosphorylation of myosin while attached to actin. Alternative theories have proposed that dephosphorylated-detached myosin can also re-attach to actin and contribute to force maintenance. The goal of this study was to investigate the role of calponin in regulating and enhancing the binding force of unphosphorylated tonic muscle myosin to actin. To confirm the calponin regulation of our actin filaments at physiological concentrations, we used the in-vitro motility assay to measure the rate ($1/2_{max}$) of actin propulsion by myosin. Our results showed a significant decrease in $1/2_{max}$ (from 0.54 ± 0.01 μm/s (mean ± SE) in the absence to 0.43 ± 0.01 μm/s in the presence of calponin). When calponin was phosphorylated, this inhibitory effect was suppressed (0.50 ± 0.01 μm/s). To measure the force of binding (F_{unb}) of unphosphorylated myosin to actin, we used the laser trap assay. F_{unb} was normalized by the number of myosin molecules estimated per actin filament length. F_{unb} of unregulated actin (0.12 ± 0.01 pN; mean ± SE) was significantly increased in the presence of calponin (0.20 ± 0.02 pN). When calponin was phosphorylated, this enhancement was lost (0.12 ± 0.01 pN). To verify whether this enhancement of F_{unb} is due to cross-linking of myosinto actin by calponin, we repeated the measurements at high [KCl], as the calponin affinity for myosin decreases at high ionic strength. Indeed, the $F_{unb,}$ in presence of calponin, obtained at a [KCl] of 25mM (0.21 ± 0.02 pN; mean ± SE) was significantly decreased at a [KCl] of 150mM, (0.13 ± 0.01 pN). These data demonstrate that calponin enhances the force of binding of unphosphorylated myosin to actin by cross-linking them together.

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Contractile Properties of Human Fetal Skeletal Myofibrils

Alice Ward Racca, Anita E. Beck, Vijay Rao, Michael Bamshad, Michael Regnier.
University of Washiington, Seattle, WA, USA.

Congenital contractures such as clubfoot are present in ~1/250 live births. Several congenital contractures syndromes are caused by mutations in genes that code for skeletal myofilament proteins, including *MYH3* and *MYH8*. Because embryonic (*MYH3*) and perinatal (*MYH8*) myosin heavy chains are unique to the prenatal development of muscle, it is important to understand the contractile properties of human embryonic (HE) myosin and human fetal (HF) muscle to determine how mutations affect performance and development. However, information on HF skeletal muscle function is lacking. We previously reported HE myosin crossbridge cycling (measured by *in vitro* motility) was much slower that rabbit psoas (RP) myosin (Biophys. J. (2010), 98:542a). Here we characterized the contraction and relaxation properties of HF muscle using myofibril mechanics techniques. HF skeletal muscle and myofibrils were isolated from a 15.4 week gestation fetus. During maximal calcium activation (15°C) HF myofibrils produced much lower force (F_{MAX}=5.9 ± 1.2mN/mm^2) as compared to myofibrils from human adult (HA) skeletal muscle (84 ± 34mN/mm^2) or RP muscle (220 ± 40mN/mm^2). Unlike control HA and RP fibrils, no striation pattern was apparent for HF myofibrils, suggesting that immature sarcomeres could explain the lower force production. HF myofibrils had slower kinetics of force development ($k_{ACT=}0.66 \pm 0.1s^{-1}$) vs. HA ($7.5 \pm 6.3s^{-1}$) and RP ($5.7 \pm 0.5s^{-1}$) myofibrils. The initial (slow) phase of relaxation upon return to low calcium solution was slower and prolonged ($k_{REL,SLOW}$=$0.59 \pm 0.22s^{-1}$; $t_{REL,SLOW}$=174 ± 13ms) vs. HA ($2.9 \pm 1.7s^{-1}$; 71 ± 18ms) or RP ($2.1 \pm 0.4s^{-1}$; 73 ± 14ms) myofibrils. The larger, faster phase of relaxation was also slower (HF=$1.5 \pm 0.2s^{-1}$ vs. HA=$12 \pm 5s^{-1}$ and RP=$21 \pm 4s^{-1}$). Our previous *in vitro* motility experiments indicated a similar inhibition of filament speed by increasing [ADP] for HE and RP myosin, but this was under low load, thus ongoing experiments will determine if ADP release is responsible for the slower kinetics of HF muscle. Funded by F31AR06300(A.R.), 5K23HD057331(A.B.), HD048895(M.B., M.R.).

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Staurosporine Blocks the ATP-Sensitive K^+Channels and Induces Atrophy in Rodent Skeletal Muscles

Sara Calzolaro, Gianluigi Cannone, Giulia Maria Camerino, Antonietta Mele, Diana Conte, Domenico Tricarico.
University of Bari, Bari, Italy.

The ATP-sensitive K^+channels(KATP) has been proposed as a molecular sensor of atrophy in skeletal muscle(Tricarico et al., 2010). In the present work we evaluated the "in vitro" effects of staurosporine, a well known apoptotic agent, on sarcolemmal KATP channels and on muscle proteins content and fibers diameter in slow-twitch *Soleus*(SOL) and fast-twitch *Exstensor Digitorum Longus*(EDL) and *Flexor Digitorum Brevis*(FDB) muscles of mice. The isolated muscles and the single fibers were incubated with staurosporine (1 μg/mL) for 1-6-24-48 hrs. alone or in combination with diazoxide(100 μM-250 μM), a KATP opener, and the changes in the total proteins content, gene expression, fibers diameter and KATP channel activity were evaluated. We found that staurosporine blocks KATP channels in excised-patch experiments being more effective in SOL rather than in EDL and FDB fibers. The toxin also caused a reduction of the proteins content/muscle of −45%, −25% and −21% for SOL, EDL and FDB, respectively at 24 hrs. A time-dependent reduction of the diameter is observed following incubation of the fibers with staurosporine. An up-regulation of the atrogin-1 gene while a down regulation of the KATP channel subunits genes is observed in SOL. The co-incubation of the muscles with staurosporine+diazoxide for 24 hrs. fully prevented the reduction of the fibers diameter and protein content, and reduction of the KATP channel activity. Our data indicate that staurosporine blocks KATP channels in skeletal muscle leading to muscle atrophy and these effects are phenotype dependent.

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Alleviation of Skeletal Muscle Defects Induced by Huntington's Disease-causing Amyloid by Modulating TOR Pathway in a Drosophila Model

Girish C. Melkani, Jennifer A. Suggs, Raul Ramos, Sanford I. Bernstein.
Department of Biology, Molecular Biology and SDSU Heart Institutes, San Diego State University, San Diego, CA, USA.

Huntington's disease (HD) is caused by an expanded polyglutamine (Poly-Q) repeat in the Huntingtin (HTT) protein. Beyond the brain, htt gene is also expressed in skeletal muscle. Muscle atrophy is common in HD patients, likely from accumulation of toxic amyloid. To explore the mechanism of Poly-Q-induced skeletal muscle defects associated with HD-causing amyloid protein, we expressed mutant HTT with short (Httex1-PolyQ25) and expanded (Httex1-PolyQ72) Poly-Q exclusively in the Drosophila indirect flight muscle (IFM). Expression of Httex1-PolyQ-72 in the IFM resulted in severe defects in skeletal muscle function and ultrastructure. Progressive reduction in flight performance was observed in 3-day and 3-week old flies (a 20% and 90% decrease in flight index, respectively) compared to age matched Httex1-PolyQ-25 flies. Further, 3-week old flies expressing Httex1-PolyQ-72 showed amyloid aggregates, whereas expression of Httex1-PolyQ-25 was benign. At the ultrastructural level, the myofibrils of control IFM showed well-organized Z-bands and M-lines, however, IFM of 3-week old flies expressing Httex1-PolyQ-72 showed severe ultrastructural deterioration with broken Z-disks, M-lines and a loss of thick filaments. In addition to myofibril defects, abnormal vacuole-like structures of various sizes and shapes containing mitochondrial remnants were present. Mutant fibers also display rimmed vacuoles fused with additional membranes, suggesting the formation of type 2 autophagic vacuoles. To further explore the mechanism underlying the PolyQ-induced skeletal muscle defects, we treated flies expressing Httex1-PolyQ-72 with rapamycin, a known suppressor of mTOR. Rapamycin-treated flies expressing Httex1-PolyQ-72 showed significantly improved flight function (a 30% increase in flight index) and improved myofibril integrity (organized Z-bands and M-lines) compared to flies expressing Httex1-PolyQ-72 without rapamycin treatment. Thus, we have developed a novel Drosophila model to explore and suppress skeletal muscle defects linked with HD-causing amyloid by modulating the TOR pathway.

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Altered Cross Bridge Kinetics in Skeletal Myofibrils from NEBΔex55, a Novel Mouse Model of Nebulin-Based Nemaline Myopathy

Claudia Ferrara[1], Danielle Buck[2], Nicoletta Piroddi[1], Chiara Tesi[1], Josine LeWinter[2], Coen Ottenheijm[2,3], Corrado Poggesi[1], Henk Granzier[3].
[1]Dip.Scienze fisiologiche, Università di Firenze, Florence, Italy, [2]Department of Physiology, VU University Medical Center, Amsterdam, Netherlands, [3]Department of Physiology, University of Arizona, Tucson, AZ, USA.